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Cloning, characterization and disruption of a (p)ppGpp synthetase gene (*relA*) of *Streptomyces coelicolor* A3(2)

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Summary

An internal segment of the (p)ppGpp synthetase gene, *relA*, of *Streptomyces coelicolor* A3(2) was amplified from genomic DNA using the polymerase chain reaction and used as a hybridization probe to isolate the complete gene from a cosmid library. *relA* lies downstream of a gene (*apt*) that apparently encodes adenine phosphoribosyltransferase and is transcribed from two promoters, *relAp1* and *relAp2*, and by transcriptional readthrough from *apt*. While the level of *relAp2* transcripts remained relatively constant, *relAp1* activity apparently peaked during transition phase, following a decline in readthrough transcription from *apt*. Disruption of *relA* using an *att*[−] derivative of the temperate phage ϕ C31 abolished ppGpp synthesis on amino acid depletion. When grown on agar, the disruptants grew more slowly than a control lysogen made with an *att*⁺ phage vector and gave smaller colonies that sporulated normally. The *relA* mutation had no consistent or marked effect on actinorhodin production in either liquid- or agar-grown cultures, indicating that elevated levels of (p)ppGpp are not essential for triggering the onset of antibiotic production.

Introduction

Streptomyces are Gram-positive mycelial soil bacteria that undergo a complex process of morphological differentiation leading to sporulation (Chater and Hopwood, 1993). They also produce a plethora of secondary metabolites, many with important applications as antibiotics in human medicine and in agriculture. Antibiotic production in streptomyces is generally confined to stationary phase in liquid cultures, and usually coincides with the onset of morphological differentiation in agar-grown cultures. While little is understood of the mechanisms that regulate antibiotic production (Champness and Chater, 1994), most of the published data are consistent with a role for growth rate,

or the cessation of growth, in determining the onset of secondary metabolism (Chater and Bibb, 1996).

In *Escherichia coli*, the highly phosphorylated guanine nucleotides ppGpp and pppGpp have been implicated in growth-rate control of gene expression (Sarubbi *et al.*, 1988; Hernandez and Bremer, 1990; 1993; Schreiber *et al.*, 1991), and in regulating stationary-phase gene expression, mediating at least some of their effects through activation of the stationary-phase sigma factor, σ^S (Gentry *et al.*, 1993). Does (p)ppGpp play a role in growth-phase-dependent gene expression in streptomyces and, more specifically, in triggering the onset of antibiotic production? Positive correlations were first observed between (p)ppGpp and antibiotic biosynthesis in *Streptomyces aureofaciens* (Simuth *et al.*, 1979) and *Streptomyces galilaeus* (Hamagishi *et al.*, 1980), producers of chlortetracycline and aclacinomycin, respectively. Later, Ochi (1986) observed an eightfold increase in formycin production by *Streptomyces lavendulae* after provoking (p)ppGpp accumulation by amino acid depletion. Moreover, a *relC* mutant of *S. lavendulae* defective in ribosomal protein L11, and consequently deficient in (p)ppGpp synthesis on amino acid starvation, was impaired in formycin production (Ochi 1986; 1990a). Similar *relC* mutants were isolated from *Streptomyces antibioticus* (Ochi, 1987), *Streptomyces griseoflavus* (Ochi, 1988), *Streptomyces griseus* (Ochi, 1990a), and *Streptomyces coelicolor* (Ochi, 1990b). The mutants accumulated only low levels of (p)ppGpp after nutritional shiftdown (on average c. 15% of wild type) and were deficient in antibiotic production, leading to the suggestion that (p)ppGpp plays a central role in triggering antibiotic biosynthesis (Ochi, 1990b). Consistent with this idea, reduced levels of actinomycin biosynthetic enzymes and mRNA were found in a *relC* mutant of *S. antibioticus* (Kelly *et al.*, 1991), and (p)ppGpp accumulation was noted to coincide with transcription of the pathway-specific activator gene, *brpA*, for bialaphos production by *Streptomyces hygroscopicus* (Holt *et al.*, 1992). However, Bascaran *et al.* (1991) concluded that (p)ppGpp did not play a role in antibiotic production in *Streptomyces clavuligerus*. Production of cephalosporins (mainly cephamycin C) occurred during a phase of slow exponential growth (doubling times of c. 7 h and 18 h in rich and minimal media, respectively), and increased in stationary phase, while ppGpp levels remained constant from the beginning of growth. *relC*-like mutants accumulated lower amounts of ppGpp than the wild-type strain after nutritional shiftdown

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(varying between 8% and 85% of the wild-type levels), but there was no simple correlation between the levels of ppGpp and cephalosporin production (some produced more, and others less, cephalosporin than the wild-type strain).

We have examined the relationship between (p)ppGpp synthesis and the production of undecylprodigiosin and actinorhodin in *S. coelicolor* (Strauch *et al.*, 1991; Takano *et al.*, 1992; Takano and Bibb, 1994). Transcription of pathway-specific activator genes for each antibiotic (*redD* for undecylprodigiosin and *actII-ORF4* for actinorhodin) conspicuously increased as the culture entered stationary phase, coinciding with a peak in the level of (p)ppGpp; this was followed by transcription of representative *red* and *act* biosynthetic structural genes, and production of the antibiotics. In contrast, transcription of the *rrnD* rRNA gene set decreased markedly (E. Takano and M. J. Bibb, unpublished). (p)ppGpp synthesis was also induced by amino acid depletion during rapid exponential growth, and was followed by an immediate and marked decrease in *rrnD* transcription (Strauch *et al.*, 1991). Transcription of *actII-ORF4* was detected within 30 min of the nutritional shift-down and transcription of the *act* biosynthetic genes after a further 30 min (Takano and Bibb, 1994).

Although correlations between (p)ppGpp synthesis and the onset of antibiotic production clearly exist, they do not establish a role for the highly phosphorylated nucleotides in triggering antibiotic biosynthesis. Although the *relC* mutants isolated by Ochi (1986; 1987; 1988; 1990a,b) show a marked reduction in antibiotic production, they grow at about half the maximal rate of the parent strain, presumably reflecting impaired protein synthesis. Thus it is difficult to assess whether the effect on antibiotic production is a direct consequence of reduced levels of (p)ppGpp, or an indirect effect of the L11 mutation on protein synthesis.

In *E. coli*, (p)ppGpp is synthesized predominantly by the ribosome-bound RelA, which is activated when an uncharged tRNA binds to the acceptor site of translating ribosomes (Cashel and Rudd, 1987). Therefore (p)ppGpp synthesis can be invoked by amino acid depletion or by addition of tRNA synthetase inhibitors, such as serine hydroxamate (SHX) (Tosa and Pizer, 1971). Interestingly, the enzyme responsible for (p)ppGpp degradation in *E. coli*, SpoT, is a homologue of RelA, the two proteins showing 31% amino acid sequence identity (Metzger *et al.*, 1989). Although the mechanism is not understood, starvation for carbon and energy reduces SpoT activity and results in elevated levels of (p)ppGpp (Cashel and Rudd, 1987). Furthermore, the residual level of (p)ppGpp synthetic activity observed in *relA* null mutants probably reflects a synthetic role for SpoT in addition to its degradative function (Hernandez and Bremer, 1991; Xiao, *et al.*, 1991), although this has not been demonstrated *in*

vitro. A *relA* homologue from *Vibrio* sp. strain S14 has been cloned, sequenced and disrupted (Flårdh *et al.*, 1994); the mutant exhibited relaxed control of RNA synthesis, and failed to accumulate (p)ppGpp upon addition of SHX. *relA*-dependent (p)ppGpp synthesis was also invoked by carbon-source depletion, which is not observed in *E. coli*; presumably glucose deprivation in the vibrio results in a rapid decrease in the level of aminoacylation of at least some tRNAs. *relA* homologues have also been identified in *Streptococcus equisimilis* H46A (Mechold *et al.*, 1993), *Mycobacterium leprae* (EMBL Accession Number U00011) and *Mycoplasma genitalium* (Peterson *et al.*, 1995); genetic and/or biochemical studies that might differentiate between synthetic and degradative roles for these genes have not been published. The isolation of *relC* mutants of *S. coelicolor* (Ochi, 1990b), and the ability of the wild-type strain to produce (p)ppGpp on amino acid depletion or on addition of SHX (Strauch *et al.*, 1991), suggests that *S. coelicolor* possesses a ribosome-dependent (p)ppGpp synthetase. In an attempt to establish if (p)ppGpp plays an essential role in triggering antibiotic production in *S. coelicolor*, we set out to isolate and disrupt its *relA* homologue.

Results

Isolation of a relA homologue from S. coelicolor using the polymerase chain reaction

Alignment of the amino acid sequences of RelA homologues from *E. coli* (RelA (Metzger *et al.*, 1988) and SpoT (Metzger *et al.*, 1989)), *M. leprae* (EMBL U00011), *S. equisimilis* H46A (Mechold *et al.*, 1993) and *Vibrio* sp. strain S14 (Flårdh *et al.*, 1994) revealed several blocks of conserved amino acids (Fig. 1). Those corresponding to amino acid residues 317–324 and 426–433 of *E. coli* RelA were chosen for the design of 16-fold and 64-fold degenerate oligonucleotides for use as primers in the polymerase chain reaction (PCR), using the preferred codons from 64 streptomycete genes (Wright and Bibb, 1992); *Bam*HI sites were incorporated at the 5' end of each primer to facilitate subsequent cloning. An amplified product of approximately 400 bp was obtained, consistent with the size expected for a *relA* homologue. The PCR product was digested with *Bam*HI and cloned in *Bam*HI-cleaved pBluescript SK+ in *E. coli* DH5 α , yielding pJ6034. Sequencing from both ends of the pJ6034 insert using universal and reverse primers revealed the expected internal segment of a *relA* homologue.

The pJ6034 insert was isolated as a *Bam*HI fragment, labelled with ³²P by random oligonucleotide priming, and used as a probe to isolate six hybridizing cosmids from a library of *S. coelicolor* M145 DNA. Digestion of each of the six cosmid DNAs with *Bgl*II, *Pvu*II, *Sac*I and

EcoRI + *Bam*HI revealed common restriction fragments, and Southern analysis using the same probe identified hybridizing *Pst*I fragments of approximately 4 kb and 6 kb. Cloning of these gel-purified *Pst*I fragments in *Pst*I-cleaved pBluescript SK+ yielded pJ6031 (4 kb insert) and pJ6037 (6 kb insert). A restriction map of the contiguous 10 kb region is shown in Fig. 2. Hybridization of the probe to cosmids L2 and L7 (H. M. Kieser, personal communication) localized the *relA* homologue on *Asel* fragment L and, more precisely, to a position between *bldD* and *argA* in the 12 o'clock region of the combined physical and genetic map of the *S. coelicolor* chromosome (Kieser *et al.*, 1992).

RelA of *S. coelicolor* has an N-terminal extension that is absent from all known homologues

The sequence of a 4515-nucleotide (nt) region that extends 2058 nt upstream and 2457 nt downstream of the *Pst*I site within *relA* was determined (EMBL X87267). Analysis of the sequence using the FRAME program (Bibb *et al.*, 1983) revealed three open reading frames (ORFs) with protein-coding character (Fig. 2). The largest of these (*relA*; nt positions 796–3336) would encode a protein of 847 amino acids (94.2 kDa) with a high degree of similarity to all of the known RelA homologues (Fig. 1). *S. coelicolor* RelA is most similar to the homologue from *M. leprae* (66% amino acid sequence identity), and least similar to RelA of *Vibrio* sp. strain 14 (38% identity), and has an N-terminal extension of approximately 90 amino acids that is absent from all of the other homologues. Unusually, translation of the *relA* transcript apparently initiates at a UUG codon (Fig. 3). Five nucleotides upstream of the UUG codon there is a sequence (AGGAG) that is complementary to a region close to the 3' end of the 16S rRNA of *S. coelicolor* (Baylis and Bibb, 1987) that is likely to serve as a ribosome-binding site.

A second ORF of 546 nt (nt positions 65 to 610) occurs upstream of, and reading in the same direction as, *relA* and is preceded 7 nt upstream by a potential ribosome-binding site (GGA). The ORF would encode a protein of 182 amino acids (18.6 kDa) with a high degree of identity to adenine phosphoribosyltransferases (APTs) from a variety of organisms. The most similar homologue found in searches of the databases was the APT of *E. coli* (51% amino acid sequence identity; Hershey and Taylor, 1986). APTs catalyse the conversion of adenine and 5-phosphoribosyl pyrophosphate to AMP in a salvage reaction that is energetically less costly than *de novo* synthesis.

The third ORF of >1095 nt is located 3' of *relA* and is oriented in the opposite direction (nt positions 3421 to 4515; Fig. 2). It terminates 86 nt from the end of the *relA* coding region; its N-terminus appears to occur outside of the sequenced region. Searches of the databases

revealed similarity (46% amino acid sequence identity in an overlap of 175 amino acids) to an ORF of unknown function from *M. leprae* (EMBL U00019) that is not located adjacent to the mycobacterial *relA* homologue. A 22 nt inverted repeat occurs in the non-coding region between *relA* and the convergent ORF (nt positions 3358–3404), and may function as a factor-independent bidirectional transcriptional terminator (reviewed in Lewin, 1994).

Transcription of relA occurs from multiple promoters in a growth-phase-dependent manner

To map the transcriptional start site of *relA*, S1 nuclease protection experiments were carried out using RNA from *S. coelicolor* strain M145 at different stages of growth. A 458 bp *Stu*I–*Sal*I fragment (nt positions 474–931) that includes the non-coding region between *apt* and *relA* (Fig. 2) was cloned in pBluescript SK+ that had been cleaved with *Sma*I and *Sal*I, yielding pJ6068. The 498 bp *Sac*I–*Sal*I fragment of pJ6068, uniquely labelled at the 5' end of the *Sal*I site (which lies 136 nt downstream of the UUG start codon) was used as probe; the presence of 40 nt of polylinker at the unlabelled end of the probe served to distinguish between probe reannealing and transcriptional readthrough from *apt*. Putative transcriptional start sites were identified at nt positions 682 and 751–752 (*relAp*1 and *relAp*2, respectively, in Figs 3 and 4b); a protected fragment corresponding in size to the *Stu*I–*Sal*I segment of the probe (Fig. 4b; FLP) indicated additional transcription of *relA* from a promoter upstream of, or within, *apt*. While the level of transcripts initiated at *relAp*2 remained constant, transcription from *relAp*1 appeared to peak during transition phase, following a marked decrease in the level of transcriptional readthrough from *apt* (Fig. 4b). An inverted repeat of nine nucleotides occurs between the *apt* and *relA* coding regions (nt positions 716–738), and overlaps the –35 region of *relAp*2 (Fig. 3).

Since the changes in the levels of the *relAp*1 and readthrough transcripts coincided with elevated levels of ppGpp during transition phase, the effect of nutritional shiftdown on *relA* transcription was assessed (Fig. 5). While there was a clear decrease in the level of the readthrough transcript 30 min after shiftdown, there was a marked increase in the amount of *relAp*1 transcript.

To confirm that the RNA-protected fragments assigned to *relAp*1 and *relAp*2 represented transcriptional start sites, rather than artefacts of S1 nuclease digestion, the 498 bp *Sac*I–*Sal*I and 466 bp *Bam*HI–*Sal*I fragments of pJ6068 were used as templates for *in vitro* transcription assays using RNA polymerase isolated from *S. coelicolor* strain M145. Both fragments gave run-off transcripts of approximately 250 nt and 180 nt (Fig. 6) that corresponded to the expected sizes predicted from the S1

Ecrela	0
Vsrela	0
Mrel	0
Screla	MPDEAQP LTA	AKPESASASA	AKPAPSAPQA	KNDTHGPIQH	APAAPVDKPA	EQQPRPKPLP	60
Ecspot	0
Serel	0
EcrelaMVAVR S AH	INKAG F FDPE	KWIASLGLITS	28
VsrelaMVAVR GA H	LKENT T FELV	SWVESLRLQDA	28
MrelMTAQ R S.T	ISPV L EPLVA	VHKEFYDPKAN	27
Screla	AERPQNAPVV	RAPAGQPARS	GSSNRVRARL	ARLGV Q EFANP	YNPV L EPLLR	IVRGNDPKIE	120
EcspotMYL F ESLNO	LIQTYLPEDQ	19	
SerelMAKE	INLT G SEVVA	LAAKYMNETD	24
Ecrela	KKSCECLAET	WAYC..LQQT	Q GHPPDASL L L	WRGVEMV E L	ST L SL S MDAD T L	RAA L L F PLAD	86
Vsrela	KVSSR.IEGT	YQRC..IELA	AEQEN G PL L L	WRGRELVE L	VT L SL S MDAD T L	IAA L L F PLV	85
Mrel	LSI...VQRA	FEVAQDRHAS	Q LKRSGDPY I	THPLAVAN I L	A ELG M DDIT L L	VA A L L HD T VE	84
Screla	TSTLRQIERA	YQVAERDAHHRG	Q KRKSGDPY I	THPLAVANT I L	A ELG M DDIT L L	MA A L L HD T VE	180
Ecspot	IKR...LRQA	YLVARDAHEG	Q TRSSGDPY I	THPVAVAC I L	A EM K L D YE T	ME A L L HD T VE	76
Serel	AAF...VKKA	LDYATAAHFY	Q V R KSGEPY I	VHPIQVAG I L	A D I HL D AV T V	AC G R I HD T VE	81
Ecrela	ANVVSSEDV L R	ESV G K S VVN L	I H G V R DMAA I	RQLKATHTDS	VSS E Q V D N V F	R N L L S A V D D F	146
Vsrela	GGCYSTDAL L S	EEYSGDEV I LHL	V QGV T EQMC A I	SQLKSTAEET	AQA A Q V D N I E	R N L L S A V D D F	145
Mrel	DTGYTLEAL S	EE F CGDEV G HL	V DGV T FLDRV	V.....LGS	..AA E GE T IE	R K IT A HA R D D F	136
Screla	DTGYTLEAL S	RDFFCDV V T	V DGV T FLDKV	K.....FGE	..AA A Q A E T IE	R K IT A HA R D D F	232
Ecspot	DIPATYQD M E	QLFGK S VAE L	V EGV S KL D KL	K.....FRD	KKE A Q A E T IE	R K IT A HA R D D F	130
Serel	DITITLDN I E	FD F GK D M V RD I	V DGV T FLG K V	E.....YKS	HEE Q L A E N H R	R K IT A HA R D D F	135
Ecrela	R CV V IK L A E R	I A H L F EVK D A	P EDERV L A A Q	E CAN I YAP L A	N FL G I G Q L K H	E LE D E L Y C F R Y I	206
Vsrela	R CV V IK L A E R	I C N L F EVK D Q	P DEVRR A A A Q	E CAN	N FL G I G Q L K H	E LE D E L Y C F R Y I	205
Mrel	R VL V IK L A D R	L H N H E T M RY L	P PE K Q A R K A F	E T L E V I A P L A	H FL G M A SV K	E LE D E L S F A I L	196
Screla	R VL V IK L A D R	L H N H E T M RY L	P PE K Q A R K A F	E T L E V I A P L A	H FL G M A SV K	E LE D E L S F A I L	292
Ecspot	R VL V IK L A D R	T H N H E T L G S L	R PD K Q R R I A E	E T L E V I A P L A	H FL G I H H I N T	E LE D E L G F E A L	190
Serel	R VL V IK L A D R	L H N H E T L K H L	R PD K Q R R I S E	E T M E I YAP L A	H FL G I S R I E	E LE D E L A E R Y L	195
Ecrela	H P T E V K R K I A K	L L H E F F L D R E	H Y I E F V G H L	R A E M K A S N V K	A E V Y Q G F P K H I	Y S I W R V H Q K K	266
Vsrela	H PD T K Q E I A K	Q L S F F L D R E	D Y I TH F V D D L	S DAM K A S N I K	A E V Y Q G F P K H I	Y S I W R V H Q K K	265
Mrel	H PK K Y E I V R	L V A G F A P S F D	T Y L AK V RA E	I STLG A S K I K	A TV E GE F P K H I	Y S I W R V H Q K K	256
Screla	Y PK M Y E I V R	L V A F A P K R D	E Y L AV T D E V	Q Q D L R A A R I K	A TV E GE F P K H I	Y S I W R V H Q K K	352
Ecspot	Y BN R FR V KE	V V K A F GN R K	E M I Q V IL S E	E GR L Q E A A G	C RV S SG H E K H L	Y S I W R V H Q K K	250
Serel	N E T E F Y K I S H	M M N E K FR E R E	A L V DD I V T K I	K S Y T T E Q G L F	G D V Y G F P E H I	Y S I W R V H Q K K	255
Ecrela	N L A F D E L F D V	R A V R I VA E R L	Q DCY A A L G V I	H THY R H L P D E	K DDY V AN P K P	N G Y Q S I H T V V	326
Vsrela	N L A F D E L F D V	R A V R I VA E R L	Q DCY A A L G V I	H TKY R H L P K E	F DDY V AN P K P	N G Y Q S I H T V V	325
Mrel	G R D F D TH I D L	V G I R I LC D E I	R DCY A A L G V I	H SLW L Q P M A G	F KDY T A P K R	G V Y Q S L H T T V	316
Screla	G R D F D TH I D L	V G I R I LC D E I	R DCY A A L G V I	H ARW N P V P G F	F KDY T A P K R	N M Y Q S L H T T V	412
Ecspot	E Q R F D HS I M D I	Y A F RV I V N D S	D TCY R V L G Q M	H SLY K PR P G F	V KDY T A P K R	N G Y Q S L H T T V	310
Serel	K K R F D Q I T F D L	I A T RC V M E T Q	S DV Y AM V G Y I	H ELW R PM P G R	F KDY T A P K R	N O Y Q S I H T T S M	315
Ecrela	L G F E G G K T V E I	Q I R T K Q M H E D	A EL G V A A H U K	Y KE G A A AG G AR S G H E D R	373
Vsrela	L GF E EG K T I E V	Q I R T K Q M H E E	S EL G V A A H U K	Y KE G TA S GG AQ S A E D E K	372
Mrel	V GP E EG K PL E V	Q I R T R K M H R T	S AE L G V A A H U K	Y K E A K AG R NG	V L H P H AA AA E I D D	367
Screla	I GP G GG K P V E V	Q I R T E DM D Q R	A AE M G V A A H U K	Y K E Q E AV A GS	K V R T D AP K SS	G K S K D HL N D	472
Ecspot	I GP H GG K P V E V	Q I R T E DM D Q R	A AE M G V A A H U K	Y K E ...H G E T ST T A D I R A	355
Serel	Y GP K S P I E I	Q I E T K EM H Q V	A AE Y G V A A H U A	Y K K G V R G K V NQ A E Q K V G	361
Ecrela	I A H L R K L I A W	Q BE E M A D S G E	M L D EV R S Q V F	D DR V Y V F T P K	G DV V D L P A G S	T P L D F A Y H I H	432
Vsrela	I N H L R K L I A W	Q BE E M S D S G E	M L D EL S R Y V L A	D DR V Y A F T P K	G DV V D L P T G S	T P L D F A Y H I H	431
Mrel	M A H L R Q L L D W	Q KE E AA E P G S E	F FL E S L R F D L S	V Q E I F V F T P K	G DV V D L P T G S	T P L D F A Y H I H	426
Screla	M A H L R Q L L D W	Q KE E AA E P G S E	F FL E S L R F D L S	R NE V E V F T P E	G D M I A L P A G A	T P L D F A Y H I H	531
Ecspot	Q R U W Q MS L LE L	Q Q S A G S S F E	F FL E S V K S D L F	P DE I Y V F T P E	G R I VE L P A G A	T P L D F A Y H I H	414
Serel	M N H L R K L I V EL	Q D A S N G D A V D	F FL V D S V K E D I F	S ER I Y V F T P T	G A V Q E L P K D S	G H T D F A Y A I H	421
Ecrela	S D V GH R C I G A	K I G GR I V P F T	Y Q L Q M G D Q I E	I I T Q K Q P N ..	P S R D V L N P N L	-G Y V T T S F G R S	490
Vsrela	S E V GH R C I G A	K V E GG L V L V E	Y HL L ENG E F V E	I I T Q K Q R P N ..	P S R D V L N P N L	-G Y V T T S F G R S	489
Mrel	T E V GH E T G A	R V N GG L V L V E	R K L ENG E F V E	I I T Q K Q R P N ..	P S R D V L N P N L	-G Y V T T S F G R S	482
Screla	T E V GH E T G A	R V N GG L V L V E	R K L ENG E F V E	I I T Q K Q R P N ..	P S R D V L N P N L	-G Y V T T S F G R S	587
Ecspot	T D I GH A C V G A	R V D ROP Y PL S	Q P L T S G Q T V E	I I T AP G A ..R	P N A A L N ..	-G Y V T T S F G R S	468
Serel	T Q V GE K A E G A	K V N GR M V L EL T	A K L K T G V V E	I V T N P N S ..FG	P S R D V L N P N L	-G Y V T T S F G R S	476
Ecrela	K I H A H FR K Q D	R D K N I L A GR Q	I L D DE L E H L G	I S L KE A E K H L L P	R Y N F N D V D E L	542
Vsrela	K I V H A FR K Q D	R D K EE A L I AG K D	I L E A E L V K I H	A T L KD A Q Y A A K H	R F N V K S P E E L	541
Mrel	K I F A H FR K Q D	R D E A E L E Q G K D	A I V RA E VR R GG	L P L Q R L V NG E	S M A A V A R E H K	Y I D V S A .. L	539
Screla	K I F A H FR K Q D	R D E A E L E Q G K D	A I V RA E VR R GG	L P I Q R L T GD	S L V T L A H EM R	Y S D I S A .. L	644
Ecspot	K I F A H FR K Q D	R D E A E L E Q G K D	A I V RA E VR R GG	L LN H A	P Q E N I Q R E L D	R M K L A T L D D L	523
Serel	K I F A H FR K Q D	R D E A E L E Q G K D	A I V RA E VR R GG	M L V S Y F Q E Q G	Y V A N K Y L D K K	K V S V K S E E S	533
Ecrela	L A A T G GG G D I R	L N Q M V N F LQ S Q F N K P S A	E E Q D A A A L K Q	L Q K S Y T P Q N	R S K D N G R .. V	597
Vsrela	V A G I G SG G D L R	I N Q V I N H IN A L V N K P T A	E E E D Q Q L L E K	L S E A S N K Q A T	S H K K P Q R D A V	598
Mrel	V A G I G SG G H V S	A R H V I V Q R I L	..Q A L G G	I D Q A E E E L E A	R S T P T .. T M L R	R Q R S T D D D V G	592
Screla	V A A I G EG H V S	A P N I V Q R K I V	..Q A L G G	E E A A T E E I D E	S V P P S R G R G R	K R R A N D A D P G	698
Ecspot	L A E I G L G N A M	S V V .. V .. A K	..Q A L G G	N L H Q H G D A S I	P A	T Q S H G H L	558
Serel	V A A I G FG G D I S	P V S V F N K I T E	..Q A L G G	A K A E A E E L V N	G G E I K H E N K D	V L K V R S H ENG	593

F L G G R A R L E P A L A G A P L E A L
 CTTCTCGGCGGGCGGCGGCTGGAGCCGGCGCTGGCCGGGGCACCGCTGGAGGCACT 600
 L T V *
 GCTCACGGTCTGAACACGCGAACCGCACGGCTGGGGCGGGCCGGAGGAATCCGGCGCC 660
 CGCCCAGGCGTTGCAGGGGTTGACGGCCGTACAGGCCCTCACATTGGCCAGAAGGCGAT 720
 CCTGGAGGGCAGGATCGCTACCATGGGGTATCCGGAGCCTGACCGGGGGACCCGGATCGC 780
 M P D E A Q P L T A A K P E S
 GCACGAGGAGTCTCTTGCCAGACGAGGCGCCAGCCACTGACCGCCGCAAGCCCGAGTCC 840

Fig. 3. Nucleotide sequence of the promoter region of *relA*. Transcription start sites for *relA* are shown by open circles, and the direction of transcription by the associated arrows. The putative -10 region of *relA*p2, and the likely ribosome-binding site and translation initiation codon of *relA* are underlined. Converging arrows indicate the inverted repeat located between *apt* and *relA*; the asterisk denotes the *apt* translation stop codon.

replaced by fragments of 5.1 kb and >40 kb, as predicted from the restriction maps of the *relA* region and of KC516. Southern analysis of the same DNA samples digested with *Pst*I again gave the pattern expected from disruption of *relA*.

All seven independent J1929::KC936 *relA* disruptants were streaked out for single colonies on R5 and minimal medium, each supplemented with $5 \mu\text{g ml}^{-1}$ Thio, together with the control lysogen J1929::KC301. All of the disruptant colonies grew more slowly than those containing KC301. All of the J1929::KC936 derivatives appeared to sporulate normally and produced the purple-pigmented antibiotic actinorhodin, but their mature colonies were significantly smaller than those of J1929::KC301. The difference in growth rate and in the size of mature colonies was also observed on R2, SFM and SMMS agar media, both in the presence and absence of $5 \mu\text{g ml}^{-1}$ Thio.

To assess the effect of disrupting *relA* on (p)ppGpp synthesis, J1929::KC936 and J1929::KC301 were grown

in SMM liquid medium containing $5 \mu\text{g ml}^{-1}$ Thio until mid-exponential phase ($\text{OD}_{450}=0.5$) and subjected to amino acid depletion by rapidly transferring the cultures to SMM lacking amino acids. Nucleotides were extracted with 1 M formic acid 0, 7.5 and 15 min after nutritional shift-down and analysed by high-performance liquid chromatography (HPLC) (Fig. 8). While the levels of ppGpp in J1929::KC301 7.5 min after amino acid depletion (542 and 292 pmol mg^{-1} dry weight in duplicate experiments) were comparable to earlier estimates for *S. coelicolor* strain M145 (200 pmol mg^{-1} dry weight; Strauch *et al.*, 1991), there was no increase in the level of ppGpp in the *relA* disruptant J1929::KC936 (7.3 and 7.8 pmol mg^{-1} dry weight in cultures with pre-shift-down ppGpp levels of 8.3 and 10.6 pmol mg^{-1} dry weight, respectively). While the level of GTP in the *relA*⁺ J1929::KC301 dropped markedly on shift-down, there was no reduction in J1929::KC936 (Fig. 8).

Growth of *S. coelicolor* in SMM is limited by the availability

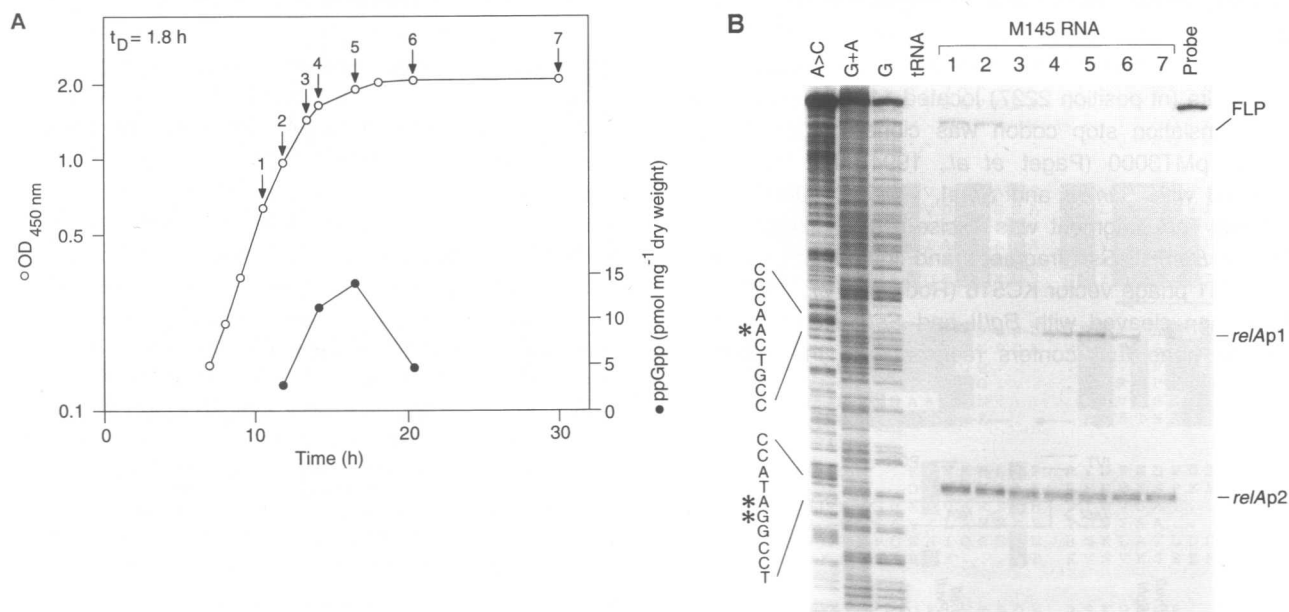


Fig. 4. A. Growth curve of *S. coelicolor* M145 with arrows showing the times at which RNA was isolated for S1 nuclease mapping. B. S1 nuclease mapping of the transcription start sites of *relA*. Asterisks indicate the probable start points of transcription; the sequences given are those of the template strand. Lanes A+C, G+A and G are sequence ladders derived from the probe using the method of Maxam and Gilbert (1980). Probe, the end-labelled fragment used as a probe; tRNA, the results obtained using $50 \mu\text{g}$ of *E. coli* tRNA; FLP, full-length protection of probe sequences homologous to the *relA* region.

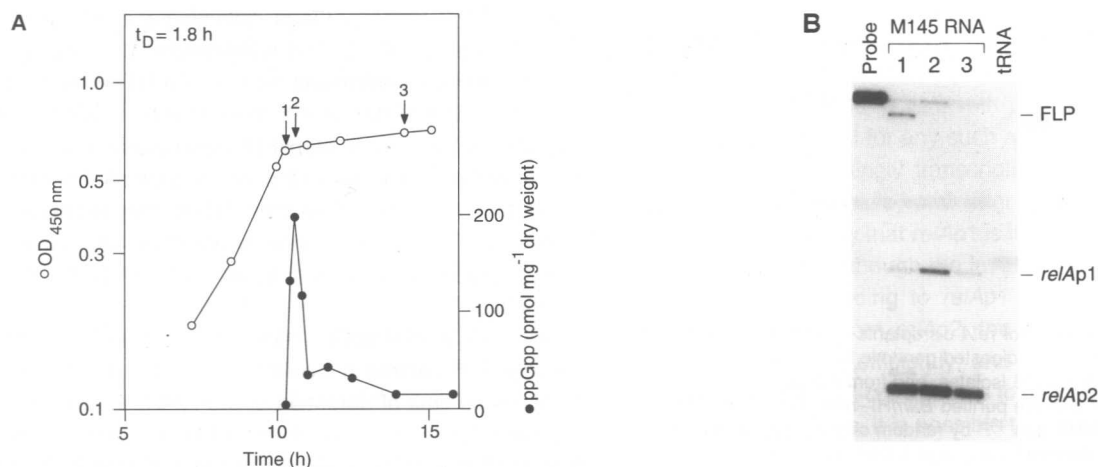


Fig. 5. A. Growth curve of *S. coelicolor* M145 after nutritional shiftdown with arrows showing the times at which RNA was isolated for S1 nuclease mapping.

B. The effect of nutritional shiftdown on *relA* transcription. See the legend to Fig. 4 for abbreviations.

of nitrogen (E. Takano and M. J. Bibb, unpublished), and (p)ppGpp synthesis occurs in *S. coelicolor* strain M145 during the transition between exponential growth and stationary phase (Strauch *et al.*, 1991). To assess the effect of the *relA* mutation on ppGpp synthesis during this transition period, ppGpp levels were monitored by HPLC. While the level of ppGpp peaked at 14.3 pmol mg⁻¹ dry weight in J1929::KC301 (similar to the 16 pmol mg⁻¹ dry weight observed by Strauch *et al.*, 1991), it remained at a constant level in J1929::KC936.

Disruption of *relA* appeared to have no deleterious effect on actinorhodin production in liquid culture, either after nutritional shiftdown or upon entry into stationary

phase. On the contrary, in both cases actinorhodin production by J1929::KC936 preceded that by J1929::KC301 by several hours. Surprisingly, in contrast to the agar-grown cultures, J1929::KC936 did not show a reduction in growth rate in liquid SMM when compared with J1929::KC301. With agar-grown cultures, although actinorhodin production in the mutant was delayed by a few hours on SMMS, there was no perceptible difference on minimal medium, R2 and R5, and on SFM it occurred several hours earlier than in the *relA*⁺ strain. The same results were obtained both in the presence and absence of Thio.

Discussion

Earlier attempts to isolate a (p)ppGpp synthetase gene from *S. coelicolor* using cloned *relA* homologues from *E. coli* (54 mol% G + C), *Serratia marcescens* (59%) and *M. leprae* (59%) as hybridization probes failed (Takano, 1993; R. Chakraborty, unpublished), presumably reflecting the considerable differences in base composition between genera (the GC content of the *S. coelicolor* *relA* homologue is 70.4%). In contrast, the PCR-based strategy reported here readily allowed for the isolation of a *relA* homologue from *S. coelicolor*; comparison of the degenerate primer sequences with the sequence of *relA* revealed 20/23 identities for the 5' primer and 23/23 for the 3' primer. Indeed, the same primers and amplification conditions gave PCR products of a similar size from *Mycobacterium smegmatis* and *Myxococcus xanthus* genomic DNAs (with GC contents deduced from the nucleotide sequence databases of 65% and 68%, respectively; R. Chakraborty, unpublished), suggesting that they may be useful for the isolation of *relA* homologues from other bacterial species with a relatively high genomic GC content.

Although the PCR product might have represented a

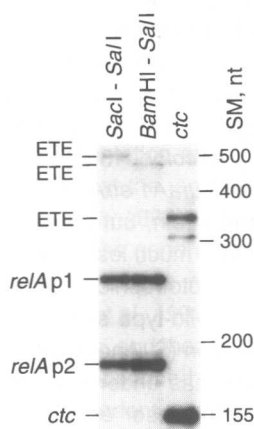


Fig. 6. *In vitro* transcription from *relAp1* and *relAp2*. The *relA* restriction fragments used as templates are shown at the top of the Figure, and the positions of run-off and end-to-end (ETE) transcripts are indicated on the right; *ctc*, a control *in vitro* transcription assay using a *ctc* promoter fragment from *Bacillus subtilis* that gives a run-off transcript of 155 nt (Buttner *et al.*, 1988). SM, size markers derived from the ³²P end-labelled 100 bp ladder and the *ctc* run-off transcript.

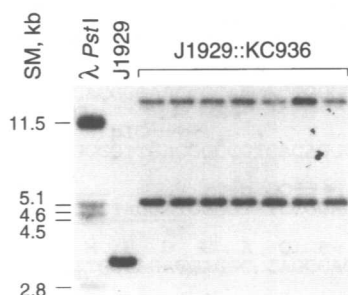


Fig. 7. Southern analysis of *relA* disruptants of *S. coelicolor* J1929. One microgram of *Bam*HI-digested genomic DNA from seven independent J1929::KC936 isolates, and from the parental strain J1929 was probed with the purified *Bam*HI-*relA*-*Pvu*II fragment that had been labelled with 32 P by random oligonucleotide priming. SM, size markers derived from phage λ DNA that had been digested with *Pst*I (the filter was also probed with phage λ DNA that had been labelled by random oligonucleotide priming).

functional homologue of *spoT* involved in (p)ppGpp degradation rather than synthesis, disruption using the *att ϕ* C31 derivative KC516 established that the *S. coelicolor* gene encodes a (p)ppGpp synthetase. ppGpp synthesis invoked by amino acid depletion in mid-exponential phase, and during the transition between exponential growth and stationary phase under conditions of nitrogen limitation, was abolished in the *relA* mutant. While ATP levels after shutdown were similar in both J1929::KC936

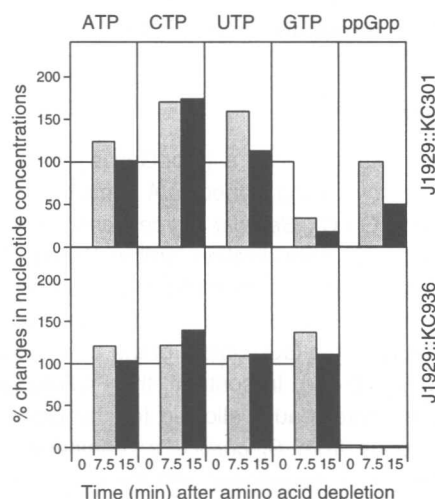


Fig. 8. HPLC analysis of ppGpp and NTP levels after amino acid depletion of J1929::KC301 (*relA*⁺) and J1929::KC936 (*relA*⁻). The results are the average values from two independent experiments. NTP levels were standardized to those observed immediately before amino acid depletion in each culture (100%); ppGpp levels were standardized to the levels observed 7.5 min after shutdown in the J1929::KC301 cultures. Nucleotide concentrations (in pmol mg⁻¹ dry weight) immediately before shutdown were: J1929::KC301 - ATP, 5668 and 5495; CTP, 490 and 647; UTP, 696 and 969; GTP, 1063 and 2056; ppGpp, >1 and 1.7. J1929::KC936 - ATP, 6347 and 4641; CTP, 520 and 752; UTP, 1468 and 1685; GTP, 1800 and 1022; ppGpp, 8.3 and 10.6.

and J1929::KC301 (perhaps reflecting the higher intracellular concentrations and wide-ranging metabolic role of ATP), the concentrations of CTP and UTP were higher in J1929::KC301 than in the mutant, presumably indicating stringent control of stable RNA synthesis in the *relA*⁺ strain. While GTP levels in the *relA* mutant after shutdown were similar to those of the other NTPs, they fell markedly in the *relA*⁺ strain (as previously noted by Strauch *et al.*, 1991), presumably reflecting, at least in part, ppGpp synthesis.

Since the level of ppGpp observed in the *relA* mutant (on average 1.7% of that in the *relA*⁺ strain after shutdown and close to the limit of detection of the HPLC assay) did not increase upon amino acid depletion, it seems unlikely that it reflects reconstitution of a functional *relA* gene by excision of KC936 (Southern analysis failed to reveal any prophage excision in cultures grown in the presence of Thio; Fig. 7). It may reflect *relA*-independent ppGpp synthesis mediated either by a functional homologue of *spoT* or by a homologue of a novel (p)ppGpp synthetase activity recently identified in *S. antibioticus* (Jones, 1994a,b; G. H. Jones, personal communication). Alternatively, the N-terminal segment of RelA that remains after disruption may possess synthetic activity that does not require ribosome activation; consequently, the level of ppGpp would not be expected to increase upon amino acid depletion, consistent with the experimental observations.

The results presented here suggest that elevated levels of (p)ppGpp are not essential for triggering actinorhodin production in *S. coelicolor*. However, since differences in the timing of actinorhodin synthesis were observed between J1929::KC936 and J1929::KC301 with some media, it is possible that variations in the level of ppGpp can influence the onset of actinorhodin production. Moreover, since the *relA* mutant still makes ppGpp, we cannot exclude the possibility that the phosphorylated nucleotide is required for transcription of antibiotic biosynthetic genes. In this context, it may be pertinent to note the effect of disrupting *relA* of *S. coelicolor* J1501 with KC936 (data not shown). J1501 (*hisA1 uraA1 strA1 Pgl⁻ SCP1⁻ SCP2⁻*) is a commonly used strain, but for reasons that are not understood it produces much less actinorhodin and undecylprodigiosin than prototrophic plasmid-free strains. It also differs from the wild-type strain in the regulation of carbon-source utilization (S. Angell, personal communication) and its genome has undergone several large deletions and duplications (Kieser *et al.*, 1992). Disruption of *relA* with KC936 reproducibly gave J1501::KC936 lysogens that were either unaffected in antibiotic production or that produced undecylprodigiosin but not actinorhodin; the two phenotypes occurred in approximately equal numbers. While the basis for this heterogeneity and for the difference from J1929 is not clear, it is possible that the metabolism of ppGpp in J1501 differs from the wild-type

strain, and that the deficiency in actinorhodin production reflects either lower basal levels or the complete absence of ppGpp in J1501::KC936 (disruptants unaffected in actinorhodin production might have undergone secondary mutations that compensate for the loss of (p)ppGpp synthesis). The isolation of further mutants of J1929 that are completely devoid of (p)ppGpp should determine whether the highly phosphorylated nucleotides play an obligate role in actinorhodin production in the wild-type strain. If they do not, then the frequently observed correlation between ppGpp synthesis and the onset of antibiotic production might simply reflect the coincidental growth-phase-dependent initiation of antibiotic synthesis and the role of ppGpp in the growth-rate control of primary metabolic genes.

Of the six fully sequenced *relA* homologues, only for *spoT* of *E. coli* is there convincing evidence for ppGpp degradative activity. Comparison of all six amino acid sequences (Fig. 1) reveals several conserved regions, but fails to identify motifs that might be used to discriminate readily between enzymes with synthetic or degradative activities, and there seems to be little conservation of gene organization around the *relA* homologues studied so far that might give a clue to function. The *apt* and *relA* genes of *S. coelicolor* are preceded by ORFs (EMBL X85969) that are homologous to *secDF* of *E. coli*; SecD and SecF appear to act to improve the efficiency of protein export, but are not absolutely required (Pogliano and Beckwith, 1994). Both *relA* and *spoT* of *E. coli* are contained within polycistronic transcription units, and neither maps close to *apt* or *secDF* (Rudd and Cashel, 1987); the *relA* homologue of *Vibrio* sp. strain S14 is preceded by a homologue of the gene found immediately upstream of *E. coli relA* (Flårdh *et al.*, 1994). The *relA* homologue of *M. leprae* is preceded by an ORF reading in the same direction (EMBL U00011), and is followed by a converging ORF, but neither encodes a protein similar to those flanking *S. coelicolor relA*. In *S. equisimilis*, the *relA* homologue is followed by a small ORF of unknown function that is transcribed in the same orientation as the putative (p)ppGpp synthetase, and then by the converging streptokinase gene, *spc* (Mechold *et al.*, 1993). Only in *M. genitalium* is there a resemblance of the gene organization found in *S. coelicolor*; the *relA* homologue of *M. genitalium* (designated *spoT* by Peterson *et al.*, 1995) is preceded by a small ORF of unknown function, and then by a homologue of *apt*.

S. coelicolor relA is transcribed from multiple promoters in a growth-phase-dependent manner. The start site for the *relAp2* transcript, which persists at a constant level throughout growth, is preceded by a -10 region (TACCAT) that is similar to the consensus sequence (TAGYYT, where Y=G or A) deduced by Strohl (1992) for streptomycete promoters that are probably transcribed by the major RNA polymerase holoenzyme (Brown *et al.*, 1992), but lacks a recognizable -35 region. Perhaps the inverted

repeat (Fig. 3) extending from -13 to -33 binds a transcriptional activator that would obviate the need for a conventional -35 sequence. However, since transcription from *relAp2* occurred *in vitro* with purified RNA polymerase, the requirement for any such activator is presumably not absolute. Interestingly, transcription from *relAp1* peaked during transition phase, and after nutritional shiftdown, raising the possibility that *relAp1* is itself subject to positive stringent control. Although the level of the RNA-protected fragment corresponding to *relAp1* was noticeably lower than that derived from *relAp2*, the longer transcript may be translated more efficiently, and while differential transcription of *relA* in *E. coli* appears to play no role in regulating ppGpp synthesis, it is possible that increased levels of *relA* transcription contribute, at least in part, to the elevated levels of ppGpp in *S. coelicolor*. Alternatively, increased levels of transcription from *relAp1* may simply compensate for the decrease in readthrough transcription from *apt*, which is presumably subject to negative stringent control, and may serve to maintain constant levels of RelA. While the sequences upstream of the *relAp1* transcription start site do not resemble any other known streptomycete promoter (Strohl, 1992), the sequence TTGCA, which starts 10-11 bases upstream of the start site, matches perfectly the consensus -12 sequence of promoters recognized by σ^{54} (Gross *et al.*, 1992); further analysis will be required to identify the RNA polymerase holoenzyme responsible for initiating transcription at *relAp1*. The significance of the transcriptional readthrough from *apt*, and its growth-phase dependence, remains to be determined.

Experimental procedures

Bacterial strains, and culture and transformation conditions

S. coelicolor strains J1929 (Bedford *et al.*, 1995) and M145 (Hopwood *et al.*, 1985), and *S. lividans* strain 1326 (Hopwood *et al.*, 1985), were manipulated as previously described (Hopwood *et al.*, 1985). SMM is the modified minimal medium of Takano *et al.* (1992); SMMS is a solidified version of SMM containing 1.5% (w/v) agar, but lacks antifoam and PEG 6000. SFM contains 2% (w/v) mannitol, 2% (w/v) soya flour and 2% (w/v) agar in tap water, and was autoclaved twice. *E. coli* K-12 DH5 α (Hanahan, 1983) was grown and transformed according to Sambrook *et al.* (1989), and transformants were selected with carbenicillin at a final concentration of 200 μ g ml⁻¹.

Isolation of plasmid DNA from *E. coli*

Plasmid DNA was isolated from *E. coli* strains using the following procedure (C. Thomas, personal communication), which reproducibly gave DNA of high purity that could be used directly for sequencing. A 10 ml culture grown overnight at 37 °C in L broth containing 200 μ g ml⁻¹ carbenicillin was harvested by centrifugation, resuspended in 1 ml of TEG (25 mM Tris pH 8, 10 mM EDTA, 50 mM glucose), and left at room

temperature for 5 min; 2 ml of 0.2 M NaOH/1% (w/v) SDS was added and the mixture was left at room temperature for 5 min. Sodium acetate (pH 4.8) (1.5 ml of 3 M) was added, and the mixture was vortexed, left on ice for 10 min, and microcentrifuged for 10 min. The supernatant was extracted with an equal volume of phenol:chloroform (1:1, v/v), and the nucleic acid precipitated by adding an equal volume of isopropanol and leaving on ice for 10 min. The pellet was harvested by centrifugation, washed in 80% (v/v) ethanol, dried, and redissolved in 1 ml of water. LiCl (333 µl of 8 M) was added and the solution was left at -20°C for at least 20 min. After centrifugation, the supernatant was transferred to a clean tube, and 70 µl of 3 M sodium acetate (pH 6) and 3.5 ml of ethanol were added. After storage at -20°C for at least 20 min, the DNA was harvested by centrifugation, washed with 70% (v/v) ethanol, and the pellet resuspended in 200 µl.

PCR

The synthetic oligonucleotides 5'-CATGGATCCAACGG-(CG)TACGAG(AT)(CG)(CG)(AC)T(CG)CACAC and 5'-CATGGATCCGTGTG(CG)A(CT)(CG)GCGTA(CG)GCGAAGTC (bracketed nt indicate positions of degeneracy) were used in the DNA PCR (Erich, 1989) to amplify an internal segment of the *relA* homologue from *S. coelicolor* M145 DNA. The reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl_2 , 0.1% (w/v) gelatin, 200 µM of each of the four dNTPs, 2.5 U of *Taq* polymerase, 40 pmol of each primer, and 50 ng of chromosomal DNA in a final volume of 100 µl. After denaturation at 95°C for 5 min, the samples were subjected to 25 cycles of denaturation (94°C , 1 min), annealing (55°C , 40 s), and extension (72°C , 50 s) and then incubated for 10 min at 72°C . PCR products were analysed by agarose (1.2% w/v) gel electrophoresis.

Nucleotide sequencing

The nucleotide sequence of the *relA* region was determined by dideoxy-sequencing (Sanger *et al.*, 1977) using the Promega TaqTrack sequencing kit and a mixture of double- and single-stranded DNA templates derived by subcloning from pIJ6031 and pIJ6037. Synthetic oligonucleotides were used to close gaps in the sequence. The region encoding *relA* and *apt* submitted to the databases (EMBL X87267) was sequenced on both strands; parts of the sequence downstream of *relA* (nt positions 3351 to 4515) were determined on only one strand and the data are available from the authors on request.

S1 nuclease protection studies

pIJ6068, pBluescript SK+ with the 458 bp *StuI*-*relA*-*SalI* fragment (nt positions 474-931) cloned between the *SmaI* and *SalI* sites of the vector, was digested with *SalI* and calf-intestinal alkaline phosphatase, and the DNA extracted with phenol:chloroform (1:1, v/v), ethanol-precipitated and digested with *SstI*. The gel-purified 498 bp *SstI*-*SalI* fragment (10 pmol) was labelled using polynucleotide kinase and 30 pmol of [γ - ^{32}P]-ATP (3000 Ci mmol $^{-1}$, Amersham) (Sambrook *et al.*, 1989). Unincorporated label was removed from the probe by passage through a Pharmacia Nick column. Fifty micrograms

of RNA, isolated from cultures of *S. coelicolor* M145 grown in SMM liquid medium (Takano *et al.*, 1992), were used in each S1 nuclease protection experiment with about 0.2 pmol (approximately 10^5 Cerenkov c.p.m.) of the probe. Hybridizations were carried out in NaTCA buffer (Murray, 1986) at 45°C overnight after denaturation at 65°C for 15 min. S1 nuclease digestions and analysis of RNA-protected fragments were carried out as previously described (Janssen *et al.*, 1989). The endpoints of the RNA-protected fragments were determined by sequencing a sample of the same end-labelled fragment that was used as probe using the method of Maxam and Gilbert (1980).

In vitro transcription

RNA polymerase was isolated from a transition-phase culture of *S. coelicolor* M145, as described by Buttner and Brown (1985), and *in vitro* run-off transcription assays were performed according to Buttner *et al.* (1987). The 498 bp *SstI*-*SalI* and 466 bp *BamHI*-*SalI* fragments of pIJ6068 were purified by gel electrophoresis and used as templates. Transcripts were analysed on denaturing 6% polyacrylamide gels using the Gibco BRL 100 bp ladder that had been end-labelled with ^{32}P as size marker.

Plaque lifts and Southern hybridization

Plaque lifts and Southern blots (Southern, 1975) were carried out as described by Hopwood *et al.* (1985), using gel-purified DNA fragments containing internal segments of *relA* labelled with ^{32}P by random oligonucleotide priming (Sambrook *et al.*, 1989).

Amino acid depletion and (p)ppGpp assays

Exponentially growing cultures ($\text{OD}_{450\text{ nm}}$ values of 0.5-0.6) were harvested by filtration (Whatman nitrocellulose filters, 0.45 µm pore size) from SMM medium containing $5\text{ }\mu\text{g ml}^{-1}$ Thio, rapidly washed with either SMM or SMM lacking cas-amino acids, resuspended in the same medium that was used for washing, and quickly returned to the shaking incubator; the 15 mM $(\text{NH}_4)_2\text{SO}_4$ present in SMM provides a source of inorganic nitrogen for the continued growth of cultures depleted of amino acids. (p)ppGpp assays were carried out as described by Strauch *et al.* (1991).

Note added in proof

We have made a *relA* null mutant of the prototrophic *S. coelicolor* strain M600 in which most of the *relA* coding region (corresponding to amino acid residues 168-686) has been deleted. On SMMS medium the mutant, which must lack *relA*-dependent ppGpp synthetase activity, does not produce γ -actinorhodin (the blue diffusible lactone form of actinorhodin; L. Bystrykh, personal communication) or actinorhodin (the purple mycelial form); on the richer R5 medium, γ -actinorhodin production is abolished, although some actinorhodin is made, while on R2 medium (R5 lacking yeast extract) there is no apparent effect on antibiotic production. We believe that the N-terminal segment of RelA that remains after the disruption reported in this paper possesses ribosome-independent

ppGpp synthetase activity, and that the elevated basal level of ppGpp observed in the disruptant (8.3 and 10.6 pmol mg⁻¹ dry weight compared with >1 and 1.7 pmol mg⁻¹ dry weight for the *relA*⁺ congenic strain) represents the constitutive synthesis of ppGpp, and accounts for the reduction in growth rate observed on solid media. These recent results also suggest that under some nutritional conditions ppGpp plays an essential role in antibiotic production in *S. coelicolor*.

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